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Fluorescence Depolarization Studies of the Phase Transition in Multilamellar Phospholipid Vesicles Exposed to 1.0-GHz Microwave Radiation

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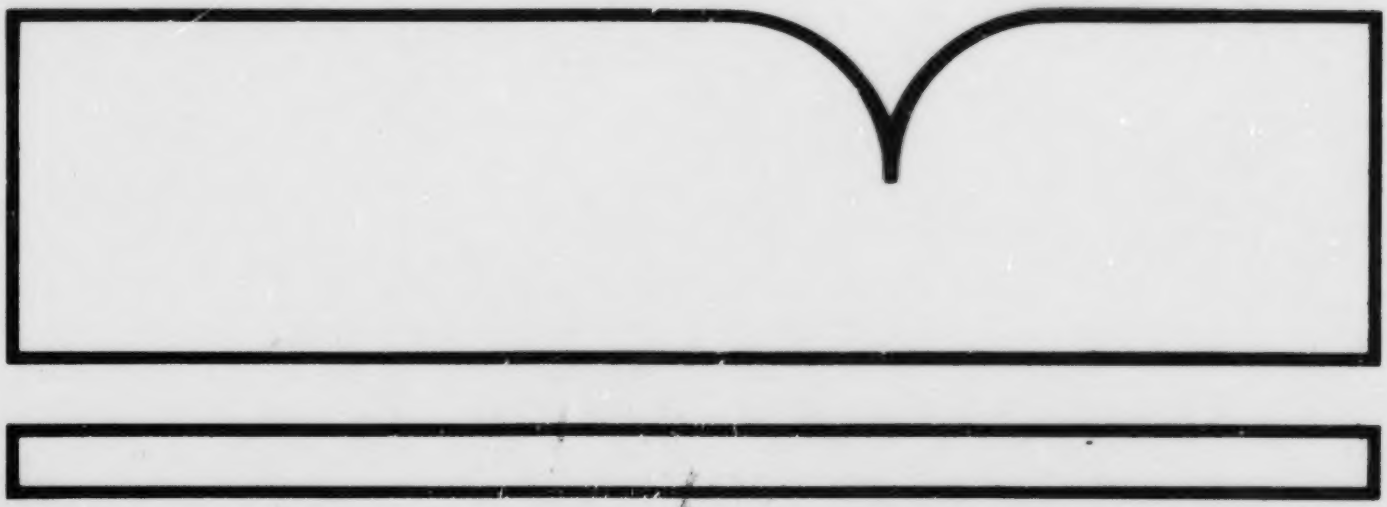

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16. ABSTRACT

The phase transition in multilamellar dimyristoylphosphatidylcholine (DMPC) vesicles was studied during exposure to continous wave 1.0-GHz microwave radiation. Fluorescence depolarization measurements using a lipid-seeking molecular probe, diphenylhexatriene (DPH), were performed as a function of temperature. Semilog plots of microviscosity versus temperature illustrate the phase transition which shows a 5 C shift when the vesicles are treated with chloroform as a positive control. No shift of the phase transition was found during exposure to microwave radiation at specific absorption rates between 1 and 30 W/kg. Samples were exposed in a rectangular transmission line (TEM cell), and specific absorption rates were calculated from electrical measurements of incident, reflected, and transmitted power. Samples were exposed to increasing intensities of radiation, while the temperature was maintained at either 23.5 or 25.5 C; these temperatures represented the two ends of the phase transition region for these vesicles. No statistically significant difference was found between exposed and control samples. These results are in contrast to those of others using laser Raman spectroscopy to measure the phase transition in similar multilamellar vesicles exposed to microwave radiation.

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Fluorescence Depolarization Studies of the Phase Transition in Multilamellar Phospholipid Vesicles Exposed to 1.0-GHz Microwave Radiation

John W. Allis and Barbara L. Sinha
Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, North Carolina

The phase transition in multilamellar dimyristoylphosphatidylcholine (DMPC) vesicles was studied during exposure to continuous wave 1.0-GHz microwave radiation. Fluorescence depolarization measurements using a lipid-seeking molecular probe, diphenylhexatriene (DPH), were performed as a function of temperature. Semilog plots of microviscosity versus temperature illustrate the phase transition which shows a 5 °C shift when the vesicles are treated with chloroform as a positive control. No shift of the phase transition was found during exposure to microwave radiation at specific absorption rates between 1 and 30 W/kg. Samples were exposed in a rectangular transmission line (TEM cell), and specific absorption rates were calculated from electrical measurements of incident, reflected, and transmitted power. Samples were exposed to increasing intensities of radiation, while the temperature was maintained at either 23.5 or 25.5 °C; these temperatures represented the two ends of the phase transition region for these vesicles. No statistically significant difference was found between exposed and control samples. These results are in contrast to those of others using laser Raman spectroscopy to measure the phase transition in similar multilamellar vesicles exposed to microwave radiation.

Key words: diphenylhexatriene, fluorescence depolarization, microviscosity, multilamellar phospholipid vesicles, phase transition, 1-GHz microwave radiation, TEM cell

INTRODUCTION

Biological membranes have been suggested by several workers as a possible site of interaction between nonionizing radiation and biological systems. Recently, data have been accumulating which support this suggestion [Seaman and Wachtel, 1978; Pickard and Barsoum, 1981; Olcerst et al, 1980; Galvin et al, 1981]. Two reports present direct evidence of changes produced by microwave exposure in biological membranes or their analogs, phospholipid vesicles. Williams et al [1975] produced opacities in the rabbit

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Address reprint requests to Dr. John W. Allis (MD-74), Experimental Biology Division, Health Effects Research Laboratory, Environmental Protection Agency, Research Triangle Park, NC 27711

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lens using an exposure of 250 mW/cm^2, but found none by slit lamp examination of lenses exposed at 165 mW/cm^2. One lens from each group was examined by electron microscopy and both showed evidence of damage to cell membranes. Sheridan et al [1979] used Raman spectroscopy to show that the gel-to-liquid-crystalline phase transition in multilamellar phospholipid vesicles occurred at a lower temperature than normal when the preparation was exposed to microwave radiation.

Multilamellar and single lamellar phospholipid vesicles are used as models for studying the properties of the lipid structure which forms the basis for biological membranes [Quinn and Chapman, 1980]. In many model membrane systems and in some natural membranes, the lipid exhibits a highly cooperative phase transition where, as temperature increases, the hydrocarbon chains in the interior of the membrane undergo a sharp decrease in the degree of order with a corresponding increase in chain mobility. Using the laser-Raman technique, Sheridan et al [1979] were able to measure the vibrational spectra of the hydrocarbon chains in the phospholipid vesicles. When multilamellar vesicles were exposed, changes occurred in the spectra which corresponded to the lipid bilayer undergoing the phase transition; however, the bulk temperature, measured simultaneously using fluorescence spectroscopy [Cavatorta et al, 1979], was less than the temperature required for the phase transition without exposure to microwaves. They were unable to find a similar effect using single lamellar vesicles.

In this paper, we describe an experiment designed to measure the shift in the phase transition, indicated by the results the Raman experiment, using the technique of fluorescence depolarization. In this approach a fluorescent molecule with known lipid-seeking properties is inserted into multilamellar vesicles. Its motion within the membrane reflects the mobility of the hydrocarbon chains of the lipid. For this experiment we have chosen the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) which is highly sensitive to the fluidity within the hydrophobic portion of the phospholipid vesicle [Shinitzky and Barenholz, 1978] and which has been used successfully to study the phase transition in multilameliar vesicles [Lentz et al, 1976].

MATERIALS AND METHODS

Fluorescent Probe

The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co) was dissolved in tetrahydrofuran (Burdick and Jackson Labs) to give a concentration of 2×10^{-3} M. For each day's experiment fresh probe solution was diluted 1:2000 with 153 mM KCl, 17 mM Tris buffer (pH 7.4), containing 15 g/100 ml sucrose; the final stock solution was 1×10^{-6} M in DPH.

Lipid Vesicles

Multilamellar dimyristoylphosphatidylcholine (DMPC) vesicles (Sigma) were prepared by dissolving the lipid in 2 ml of chloroform (J.T. Baker Chemical Co) and placing the solution in a 50-ml round-bottom flask attached to a rotating flash evaporator (Rotavapor R110, Buchi). The flask was suspended in a 30 °C water bath and rotated for 3–4 h under vacuum. As the chloroform evaporated, a thin film of lipid was left on the walls of the round-bottom flask. Sufficient KCl–Tris–sucrose buffer was added to bring the final concentration to 5 mg lipid/ml buffer. The round-bottom flask was reattached to the rotator, and the suspension swirled in a 30 °C water bath for an additional 3–4 h to form the vesicles. They were subsequently stored at 3 °C and used within 1 week. Before each day's experiment, the vesicles were swirled at 30 °C for 45 min.

The fluorescent probe, DPH, was introduced into the DMPC membranes on the day of the experiment by mixing equal volumes of the 1×10^{-6} M DPH solution and a 1:24 dilution (KCl–Tris–sucrose buffer) of the membrane suspension. The resulting mixture was incubated at 37 °C for 60 min to facilitate penetration of the lipid membrane by the probe.

Non-probe-containing suspensions were prepared by substituting KCl–Tris–sucrose buffer for the DPH solution. This suspension was also incubated at 37 °C for 60 min and used as the light-scattering blank for the fluorescence measurements

Chloroform-treated vesicles were prepared as above except that 30 μl of chloroform were added to 9.6 ml of vesicle suspension before the 60-min incubation.

Fluorescence Measurements

Fluorescence depolarization of DPH was measured by a method similar to that described by Shinitzky and Barenholz [1974] using both Perkin Elmer MPF-44 and MPF-44B spectrofluorometers. Fluorescence intensity was measured in planes parallel and perpendicular to that of the exciting light.

The depolarization data for DPH was obtained using an excitation wavelength of 360 nm and an emission wavelength of 425 nm. The measurements were converted to apparent microviscosity of the interior of the DMPC bilayer by the method described elsewhere [Allis and Sinha, 1981].

One set of measurements was conducted as a function of temperature. Two other sets were conducted as a function of duration of microwave exposure at a fixed temperature (23.5 and 25.5 °C, respectively). The last set of measurements was conducted with the temperature set at 23.5 °C, but the SARs were increased during the experiment from ~1 mW/g at 1 h to ~25 mW/g at 5 h.

Two pairs of samples were used for fluorescence measurements. Each pair consisted of a cuvette containing the membrane suspension with DPH and another cuvette containing the membrane suspension only. Exactly 3.5 ml were measured into each cuvette. One pair was exposed to the microwave radiation and the second pair was the unexposed control. The control pair was placed in the Perkin Elmer MPF-44B sample holder which is temperature regulated by a circulating water bath. After depolarization measurements were performed on the exposed pair, the polarizing filters were switched to the MPF-44B, and depolarization measurements were taken of the control pair. For each measurement the sample fluorescence was corrected for the light-scattering contribution of the vesicles by subtracting the value of the blank from the corresponding value of the sample.

Microwave Equipment and Dose Measurement

The microwave exposure equipment is identical to that described previously [Allis and Sinha, 1981]. It is based on a small rectangular transmission line (TEM cell), mounted vertically at the front of the MPF-44 fluorometer, and is equipped with special optics so that fluorescence measurements can be performed on a sample in the TEM cell during exposure. The rates of energy absorption by the sample and blank were determined from readings of incident, transmitted and reflected power, also as described previously [Allis and Sinha, 1981].

Temperature Measurement and Control

Temperature of the exposed membrane suspensions was maintained inside the TEM cell by circulating water from an external temperature bath through thin-walled Tygon tubing wrapped around the Crawford cell. In this manner temperature of the exposed

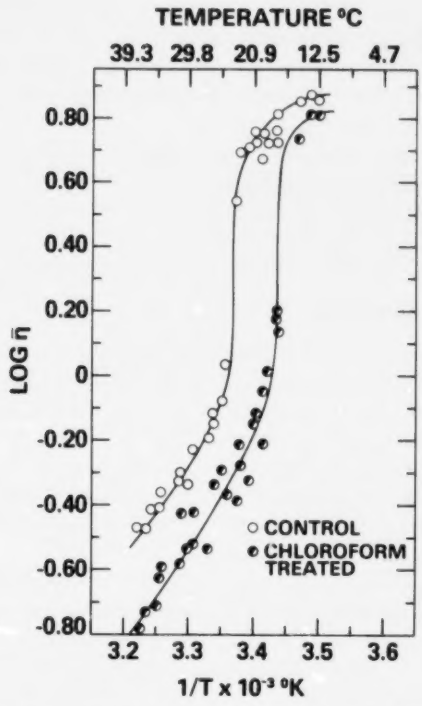

Fig. 1. Arrhenius plot of microviscosity vs temperature for the phase transition in untreated multilamellar DMPC vesicles and chloroform-treated vesicles. Samples contained 0.1 mg DMPC/ml buffer. Chloroform concentration in treated vesicle suspensions was 38.7 mM.

cuvettes was maintained within ±0.2 °C (for an individual fluorescence measurement) and was adjustable over the range 15–40 °C. The control pair was maintained at the same temperature as the exposed pair in the TEM cell.

Most of the temperature measurements were performed using a telethermometer (Yellow Springs Instruments, Model 46TUC) and a nonmetallic thermistor probe (YSI, Model 520). Temperature of the exposed and control samples were monitored as described previously [Allis and Sinha, 1981]. A Vitek probe (Model 101) became available to us near the end of these experiments and was substituted for the YSI equipment for some of the measurements.

RESULTS

The motion of the fluorescent probe, DPH, in the lipid vesicle reflects the state of the hydrophobic interior of the vesicle. As the lipid undergoes transition from the liquid-crystalline to the gel state, the hydrophobic interior of the vesicle bilayer becomes highly ordered. Consequently, the hydrocarbon chains of the lipid as well as the dissolved DPH molecules have much less opportunity for motion. This change is readily detected by

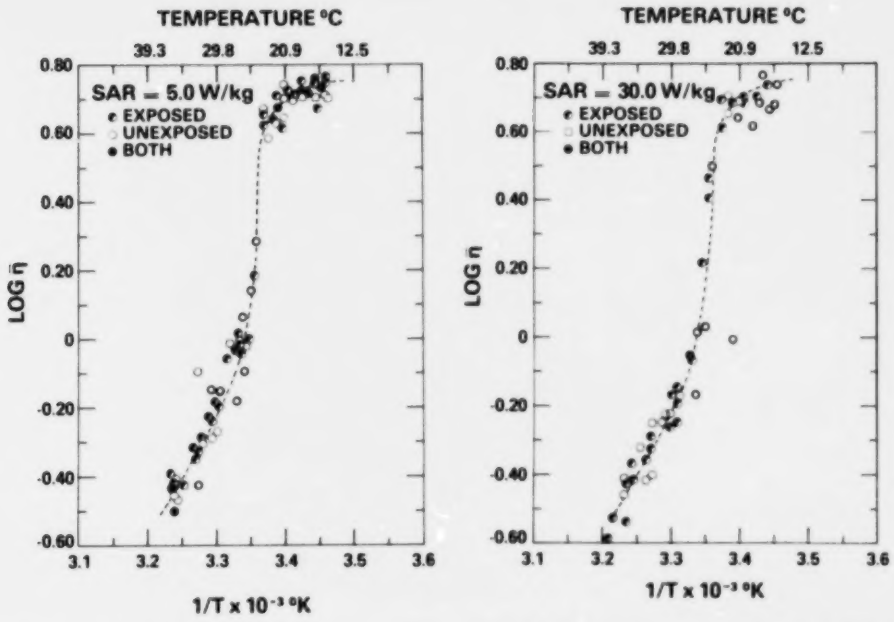

Fig. 2. Arrhenius plot of microviscosity vs temperature for the phase transition of multilamellar DMPC vesicles exposed to 1.0-GHz microwave radiation. Samples contained 0.1 mg DMPC/ml buffer. Measurements of treated samples performed during exposure at SAR indicated.

measuring the depolarization of fluorescence of the DPH. Depolarization measurements can be converted to a microviscosity, $\overline{\eta}$, as measured by the probe in the lipid bilayer (see Allis and Sinha [1981] for a brief description or Lentz et al [1976] and Shinitzky and Barenholz [1978] for a detailed discussion).

When the logarithm of the microviscosity is plotted against reciprocal temperature (Arrhenius plot), a straight line is obtained where there is no phase transition. The measurements presented here show a major transition between two linear regions, ie the liquid-crystalline to gel phase transition. In Figure 1 data for untreated DMPC vesicles are presented with the corresponding curve for DMPC vesicles treated with chloroform. Chloroform is one of several chemicals that shifts the transition to a lower temperature [Vanderkooi et al, 1977]. As can be seen in the figure, the transition temperature has been decreased by approximately 5 °C without affecting the shape of the curve substantially. Sheridan et al [1979] have indicated that their results correspond to a decrease in the transition temperature of about 2 °C. A change of about 2 °C in the transition temperature would be detectable by our technique.

Fluorescence depolarization measurements were made as a function of temperature on control samples and samples exposed to 1.0 GHz at a specific absorption rate (SAR) of 1, 5, 15, or 30 W/kg. The results of two exposure levels are shown in Figure 2A,B. There is no difference between the temperature profiles of the control vs exposed samples. Results for the other exposure levels were identical. The shift of the phase transition that would be expected on the basis of the work by Sheridan et al [1979] was not found.

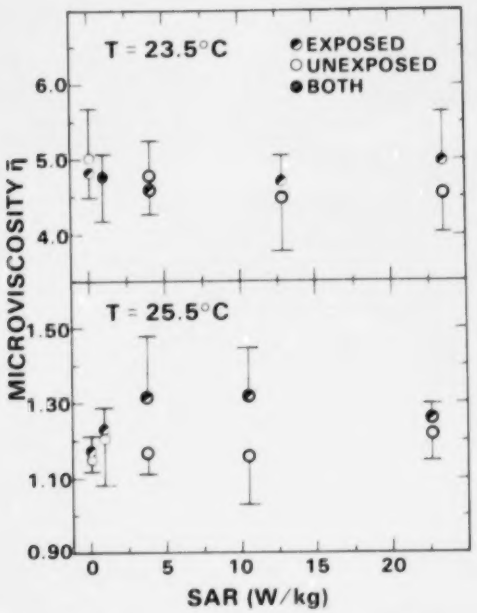

Fig. 3. Microviscosity of multilamellar DMPC vesicles exposed to successively increasing levels of 1.0-GHz microwave radiation compared to controls. The temperatures selected represent the two extremes of the phase transition region determined from Figures 1 and 2.

Measurements were also performed at constant temperature over a series of power levels. Two temperatures were chosen, 23.5 °C (in Figure 2, log $\overline{\eta}$ is about 0.7) and 25.5 °C (log $\overline{\eta}$ is about zero). These temperatures are near the breaks in the curve which bound the region of the most rapid change and were selected as points where shifts in the transition could be detected easily. The results are presented in Figure 3. Repeated measures analysis of variance for each temperature showed no statistically significant difference between the control and exposed samples as SAR was increased (at 23.5 °C, $F[4,24] = 1.35$; at 25.5 °C, $F[4,24] = 1.47$).

In order to obtain the data illustrated above, it was necessary to expose a single sample for several hours on a given day. Therefore, an additional set of control measurements was performed. A single sample was exposed for 5 h per day with measurements made each hour, and compared to an unexposed control maintained at the same temperature. These measurements were performed at four exposure levels between 1 and 25 W/kg at each of two temperatures, 23.5 and 25.5 °C. Two examples of these measurements are illustrated in Figure 4. In each case the analysis of variance showed no statistically significant trend over time of exposure.

DISCUSSION

This experiment was designed to investigate the interaction of microwave radiation with an important property of biological membranes, the internal viscosity (or its converse,

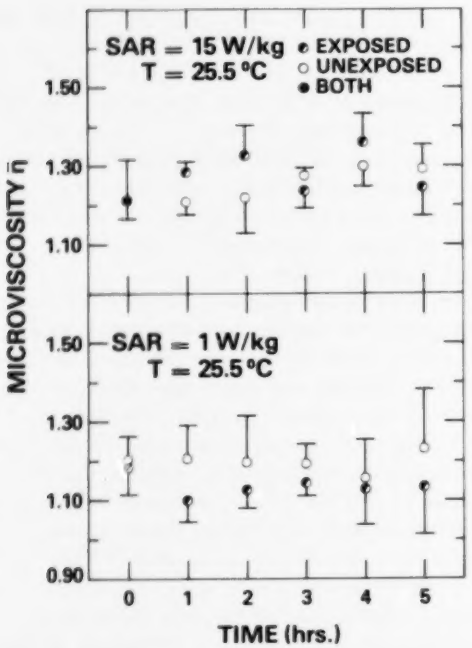

Fig. 4. Microviscosity of multilamellar DMPC vesicles exposed for 5 h at 1.0 GHz. Measurements were taken prior to beginning exposure and each hour thereafter.

fluidity) of the lipid bilayer. We also hoped to corroborate the results of Sheridan et al [1979], obtained using Raman spectroscopy. There are two general ways in which one might expect microwave radiation to interact with a lipid bilayer, and presumably with the lipid in a biological membrane. One is a direct interaction of the microwave field with the lipid molecules in the membrane. The other is an indirect interaction through the water associated with the membrane lipid. Either mechanism could explain the Raman results by producing a higher degree of motion in the lipid than would be expected from the system's (bulk) temperature.

A direct effect of microwave radiation on the membrane lipid is most likely through interaction of the oscillating electric field with the dipolar head group of the lipid molecule. The transition from gel to the liquid-crystalline phase in vesicles is characterized by an increase in the average surface area of the membrane occupied by each molecule as well as an increase in the motion of the hydrocarbon chains of the interior. An increase in the motion of the head group of the lipid molecule might be sufficient for an early initiation of the phase transition. However, because of the packing constraints in the membrane, one might expect that a frequency considerably lower than that used in either experiment might be more effective [Kaatze et al, 1979].

The water in contact with membrane surfaces is generally regarded to be of a more structured nature than normal "bulk" water. No matter what the molecular details, this would result in a lower degree of rotational freedom than in bulk water. The characteristic

interaction frequency would be significantly lower than 18 GHz, the characteristic frequency for the well-known interaction with bulk water. Some workers [Grant et al, 1978] have suggested that characteristic frequencies are as low as 300–500 MHz. Additional energy acquired by structured water at the membrane surface would have to be transmitted by molecular collision to the membrane lipid to effect a change in the lipid phase transition. This situation could conceivably be maximized in multilamellar liposomes where thin layers of water are trapped between successive lipid bilayers. Not only is there a relatively high amount of structured water, but it is compartmentalized and isolated from the solvent. Energy taken from the microwave field would have to be passed to the lipid as the first step in its eventual dissipation.

Recently, there has been some conjecture, but so far no firm evidence, that a small amount of water is trapped in the interior of the membrane. This water would be highly immobilized due to its hydrophobic environment. It would also present another site for interaction for microwave radiation, and any energy absorbed from the field would be transmitted to the hydrocarbon chains of the lipid molecules.

Although either of these mechanisms could explain the results obtained by Sheridan et al [1979], we were unable to demonstrate their occurrence in our experiments. However, we did obtain the expected response from several control experiments. Our temperature profile data for unexposed samples, which characterizes the phase transition in multilamellar DMPC vesicles, agree closely with Lentz et al [1976]. The shift of 5 °C in the phase transition produced by treating the vesicles with chloroform shows that a 2 °C shift, found in the Raman experiment, should have been detected. We also conducted measurements at constant temperature chosen to position the system at the brink of the phase transition, then exposed the samples to increasing doses of microwave radiation; this procedure simulated the Raman experiment as closely as possible using our technique. No evidence for perturbation of the lipid phase transition was found by either procedure.

The reason for the difference between the results of Sheridan et al [1979] and our own is not clear. The two methods of measurement differ in several experimental details. The most obvious, frequency of exposure, (1.0 vs 2.45 GHz), was dictated by the limitations of our equipment. This frequency difference is not likely to be significant, however, because an absorption in this range is expected to be broad and would likely encompass these two frequencies.

We have calculated SAR and electric field intensity in the sample for our experiment and for the Raman experiment (J.P. Sheridan, Naval Research Laboratory, Washington DC, personal communication). The SAR range is 1–30 W/kg, and the electric field intensities are 8–250 V/m for the fluorescence study; and 9–22 W/kg and 190–490 V/m, respectively, for the Raman study. These data are comparable and do not appear to explain the difference in results.

We chose DMPC vesicles with a temperature phase transition at about 24 °C rather than DPPC (about 41 °C) because of limitations in the range of our temperature regulation during microwave exposure. This difference is also not likely to be significant because vesicles from these lipids are very similar structurally and are known to have very similar properties near their phase transition [Quinn and Chapman, 1980].

A major difference in techniques is that Raman spectroscopy is noninvasive and measures the properties of the lipid directly. Fluorescence requires (in this case) the introduction of a probe molecule which necessarily perturbs the lipid bilayer. DPH is a slender molecule, roughly the length of the hydrocarbon chains in DMPC, and presumably positions itself parallel to these hydrocarbon chains in the lipid bilayer. The probe is not attached to any other molecule and diffuses throughout the lipid bilayer, rotating prin-

cipally about its long axis. The fluorescence depolarization technique effectively measures the degree of rotation undergone by the probe molecule between absorption and emission of the light. Thus, the probe's motion reflects the motion of the lipid molecules by being able to rotate more freely when the lipid motion is relatively rapid and less constraining. This indirect measure of the behavior of the lipid molecules near the probe may not be as sensitive as the Raman measurements to microwave-induced change. However, measurement of the unperturbed phase transition using fluorescence depolarization of DPH does accurately reflect results obtained with noninvasive techniques such as differential scanning calorimetry [Lentz et al, 1976; Shinitzky and Barenholz, 1978] and laser-Raman spectroscopy [Spiker and Levin, 1976].

The Raman technique requires a high concentration of lipid vesicles in the sample, while fluorescence requires a low concentration. Lipid concentration in our experiment was about 0.1 mg/ml, while the concentration in the Raman experiment was about 10^3 times higher or near 100 mg/ml (B.P. Gaber, Naval Research Laboratory, Washington DC, personal communication). The phase transition is affected when the water content of vesicle suspensions becomes very low [Quinn and Chapman, 1980]; however, these effects do not appear until the water content becomes 5 to 10 times lower than those used in the Raman study. In any case, there may still be some effect of the close packing of vesicles on the Raman-microwave experiment.

CONCLUSIONS

The phase transition in DMPC vesicles detected using fluorescence depolarization of DPH is identical to that obtained by others. A 5 °C shift in the transition was produced by introducing 38.7-mM chloroform into the vesicle suspension. We have been unable to corroborate the results of Sheridan et al [1979] which predicted that microwave radiation would shift the transition by 2 °C. Differences in methodology or experimental details may be responsible for this discrepancy. Laser-Raman spectroscopy may be more sensitive to the microwave effect, because it is not an invasive technique such as described in this paper. Or, perhaps the close packing of the vesicles in the Raman experiment creates an interaction which is not present when the vesicles are well separated as in the fluorescence experiment. We are continuing our efforts to investigate the properties of the lipid and the water associated with it in lipid bilayer vesicles in order to establish a possible interaction with microwave radiation.

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DISCLAIMER

This paper has been reviewed by the Health Effects Research Laboratory, United States Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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